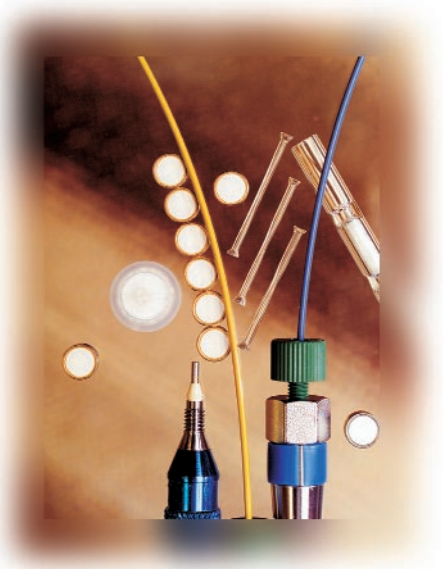
Advances in Amino Acid Analysis



The authors briefly review current strategies of amino acid analysis and present a novel approach to a simplified sample preparation of body fluids. Their procedure involves two consecutive extraction steps: a solid-phase step and a liquid–liquid step, during which the analytes are selectively isolated and rapidly alkylated while being transferred into an organic phase with a high degree of purity. The total time of sample preparation and gas chromatography (GC) analysis is approximately 15 min. The derivatives can be analyzed by GC with flame ionization, nitrogen–phosphorus, or mass spectrometry detection or by liquid chromatography with mass spectrometry detection, all of which give this procedure a versatility lacking in other current techniques.

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Every new advancement in separation techniques subsequently is applied in the bioanalytical field, in which the class of protein amino acids usually is first to be examined. Because of large differences in their chemical structures, which span from nonpolar to highly polar side chains, amino acids have always presented an analytical challenge in terms of sample preparation, separation, and detection. Numerous analytical methods have been developed in the past 50 years, and researchers have made impressive achievements in the fields of derivatization, separation, and detection of this compound class.

The ion-exchange-based separation of amino acids, followed by postcolumn ninhydrin detection, was introduced into common use in the 1950s and has remained important. Starting in the mid-1950s, gas chromatography (GC) analysis methods dominated the field for two decades. Derivatization approaches were developed with the purpose of increasing analyte volatility by removing as many active hydrogen atoms as possible from functional groups in amino acid molecules. Thereafter, the significant progress made in reversed-phase liquid chromatography (LC) stationary-phase development and in the new field of precolumn derivatization provided improved separations of analyte derivatives. Several methods proved reliable enough to address body fluid amino acid profiling in routine clinical analysis (1,2).

As analysts started using capillary electrophoresis (CE) in the 1990s, they were able to successfully use most of the popular reagents from LC analyses in electromigration techniques. The remarkable progress in detection technologies — such as diode-array ultraviolet (UV), laser-induced fluorescence, evaporative light-scattering, and mass spectrometry (MS) detection with various ionization principles — provided new opportunities in amino acid determination. The advances in evaporative light-scattering and MS techniques restored new interest in native amino acid analyses.

Despite the remarkable improvements in separation and detection technology, sample preparation before analysis remains an integral and often the most time-consuming part of the methodology. Because of the matrix complexity, preparation of biological samples such as body fluids usually is difficult and laborious, so that even excellent separation and detection methods may fail to deliver the performance and ruggedness required for routine use. This fact is recognized more often in application laboratories but seldom in the academic community. Expedient sample preparation, therefore, is of primary importance in routine applications, and the lack of it is the possible reason that a limited number of robust, user-friendly methods for analyzing amino acids

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in body fluids survive in laboratories. Without laying a claim to completeness, we will present a brief review of some viable past and present procedures for amino acid analysis to offer basic orientation in the field.

A Retrospective of Amino Acid Analysis by GC

GC analysis of polar compounds such as amino acids is fully dependent upon the availability of efficient derivatization reactions. Those reactions should generate derivatives that are less susceptible to hydrogen-bonding interactions, which deteriorate the chromatographic process. A comprehensive publication in the late 1980s summarized the preceding decades' work in the field of amino acid derivatization for GC analysis (3). Two approaches were most promising: one was based upon two-step, two-medium esterification–acylation procedures and the other was based upon one-step silylation. Although both approaches enabled protein and nonprotein amino acids to be analyzed readily (3,4), analysts encountered limitations in derivative formation. The treatments were time consuming and required water-free environments and high reaction temperatures in both cases. With procedures based upon silylation, Molnár-Perl and Katona (5) reported multiple and unstable derivatives, especially for basic amino acids. Nevertheless, researchers were successful in analyzing amino acids — mostly in the form of trifluoroacetyl butyl or heptafluorobutyryl isobutyl esters — by using either packed or capillary columns for GC analysis (1–3). Multiple-step procedures that use three different reagents for the treatment of amino, carboxylic, and alcoholic groups find followers even today (6).

In retrospect, two derivatization approaches for amino acid analysis by GC also seem worthy of notice because of their simplicity and speed. One is based upon the simultaneous treatment of α-amino and carboxylic groups with 1,3-dichlorotetrafluoroacetone, followed by the acylation of side-chain groups in the same aprotic medium (7). Hušek determined 20 protein amino acids in approximately 30 min, including the derivatization time. Another method is based upon modifying amino groups with isobutyl chloroformate in aqueous carbonate buffer, followed by the esterification of carboxylic groups with diazomethane in the ethereal extract created by the first step (8). The derivatization and capillary GC analysis time also were approximately 30 min. No sample cleanup procedure such as deproteinization or solid-phase extraction (SPE) was necessary to profile amino acids in body fluids (urine and serum) using nitrogen–phosphorus detection. This procedure did not exclude other serum organic acids from the sample; their derivatives were ignored only by the use of the nitrogen-selective detection.

Single-step derivatization of amino acids with simple alkyl chloroformates in aqueous media represents the latest advancement in derivative formation before GC analysis (9). We will demonstrate in this article that this approach is compatible with simple preparation of complex biological matrices based upon novel microscale ion-exchange SPE.

Common and Novel Procedures in LC Amino Acid Analysis

In LC, precolumn derivatization is preferable to postcolumn treatment because it ensures an easier reversed-phase separation of the less-polar derivatives compared with native amino acids. The derivatization targets the amino groups almost exclusively by attaching bulky aryl groups that enable detection by UV absorbance, fluorescence, or voltammetry. The carboxylic groups remain free to aid in the separation process. Many sound procedures using popular reagents such as *o*-phthaldialdehyde, phenylisothiocyanate, 9-fluorenylmethyl chloroformate, and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and the ability to automate the process make reversed-phase LC the most common method in amino acid analysis. However, no method is clearly preferable, especially for body fluid amino acid profiling.

With the exception of one remarkable study of plasma amino acids using *o*-phthaldialdehyde with a 12-min run time (10), general analysis times of 30–90 min are common, imino acids elude determination, and some derivatives are unstable. Reports about urine amino acid profiling are very scarce (1,11–14). *o*-Phthaldialdehyde in combination with a chiral thiol led to a prominent separation of amino acid enantiomers (15), and, combined with 9-fluorenylmethyl chloroformate, it enabled the analysis of both primary and secondary amino acids (16). Fluorescence detection (11,12) is the most widespread and sensitive detection method in use, but electrochemical (17) and MS detection (18) have proved to be viable options.

Another aldehyde (naphthalene-2,3-dicarboxaldehyde [19]) and phenylisothio-

cyanate (20) worked well for analyzing physiological amino acids. The latter reagent reacted advantageously with both amino and imino groups and provided short analysis times of 12 min for protein hydrolysates and 20 min for physiological amino acids with simple UV detection (21). However, this method required vacuum evaporation before LC analysis, so the reagent was later replaced by 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate in the automated analyzers supplied by Waters Corp. (Milford, Massachusetts). Next, researchers found that 9-fluorenylmethyl chloroformate and several other aryl chloroformates readily reacted with amino and imino groups, and they published numerous papers about analyzing protein amino acids using UV or fluorescence detection (22–26). Applications for body fluid amino acid profiling are rather exceptional, however.

Current LC attempts focus on separating underivatized amino acids using evaporative light-scattering or MS detection. Using evaporative light-scattering detection, analysts reported detection limits in the 10–20 ng range and analysis times of 40–80 min, which were comparable to the times obtained with common LC procedures that used derivatives and UV or fluorescence detection (27,28). Derivatization is preferable with MS, particularly with complex biological matrices, for several reasons: to improve detection limits, minimize ion suppression effects, and minimize adduct formation (29). Gartenmann and Kochbar (30) observed a strongly increased electrospray ionization MS response with the 9-fluorenylmethyl chloroformate derivative. Van Eijk and co-workers (18) observed a similar response with *o*-phthaldialdehyde–treated amino acids in pig plasma amino acid profiling. Likewise, Casetta and colleagues (31) found the butylation of amino acids useful in LC–MS–MS for consistent ionization efficiency with plasma samples, which were subjected to ionization following protein precipitation. The assets and liabilities of the LC–MS technique and its application fields were well summarized in two up-to-date reviews (29,32).

Current Approaches to Amino Acid Analysis by CE

Analytical chemists anticipated, and partially accomplished, fast CE separations of amino acids with minimal or even no sample pretreatment during the past decade. Oguri (33) recently published a useful review about the topic. Unlike LC, CE proved to be much more tolerant of biological fluids; occasionally a simple dilution was sufficient before injection (34). However, those approaches were not intended for complete profiling but only for targeted groups or individual amino acids.

Of the many papers published about CE amino acid determination during the past decade, a few described separations of the whole class of protein amino acids, more or less, either in native or derivative form. The latter amino acids were subjected mostly to micellar electrokinetic chromatography (MEKC) with UV-absorbance or laser-induced fluorescence detection. The separation of phenylthiohydantoin-amino acids, which are the end products of Edman protein degradation with phenylisothiocyanate, is the most rapid and successful procedure by CE (35), capillary electrochromatography (CEC) (36), and LC (37), because workers can obtain analysis times of 14 min with CE and CEC and 7 min with LC.

Napthalene-2,3-dicarboxaldehyde–mediated derivatization resulted in a remarkable separation of 14 amino acids by

MEKC in less than 30 min (38). Skocir and Prosek (39) obtained similar results with dansylated amino acids, and Hu and Li (40) were able to separate 15 4-fluoro-7-nitro-2,1,3-benzoxadiazole–treated amino acids, except for some critical pairs, in 22 min. Using the combined action of *o*-phthaldialdehyde and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose, Tivesten and Folestad (41) achieved a remarkable degree of separation for 14 amino acid enantiomers on a short capillary column in less than 5 min.

Advances in microchip-channel fabrication have allowed even shorter analysis times and better resolutions, as Culbertson and co-workers (42) reported about their separation of 19 tetramethylrhodamine-labeled amino acids in less than 3 min. Although most of these publications dealt with the analysis of amino acid standards, very few focused on body fluid amino acid profiling. Among those few publications, one concerned the screening of amino acids and amines in a 50-μL cerebrospinal fluid sample labeled with fluorescein isothiocyanate, and this separation recorded more than 50 peaks in less than 22 min (43). In another study, Thorsen and Berquist (44) used MEKC for the first time for the chiral analysis of amino acids in urine and cerebrospinal fluid after derivatization with a chiral aryl chloroformate. Boulat and coworkers (45) labeled primary amino acids with 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde in 40 min, which enabled them to separate 29 amino acid standards and plasma amino acids in 70 min. They used the procedure for a semiquantitative diagnosis of several inborn errors of metabolism (45). In most of these studies, laser-induced fluorescence detection proved to be the most successful detection method for overcoming CE's lack of concentration and detection sensitivity.

Analysts have attempted to analze underivatized amino acids far more frequently when using CE than when using LC. Unfortunately, MEKC is rather incompatible with MS because of the use of nonvolatile ion-pairing surfactants in the mobile phase. Electrooxidation of amino acids and peptides on copper electrodes proved useful in urinary amino acid quantification by coupling CE with electrochemical detection (46). Using either indirect (47,48) or direct (49) UV-absorbance detection, researchers were able to achieve separations of most protein amino acids in relatively short analysis times of 15–25 min.

Recent top reports (50,51) are based upon advances in the hyphenated CE–MS technology (32). Native amino acids were determined quantitatively in child plasma (50) and in urine in less than 17 min (51) with a surprising degree of reproducibility. The deproteinization of samples before analysis required more than 1 h, however, so that the time gained by nonderivatization was negated (50). Soga and Heiger (51) found that CE analysis of underivatized amino acids with electrospray ionization MS detection was approximately 100-fold more sensitive than analysis using indirect UV detection, but it also was 10- to 100-fold less sensitive than fluorescence detection of *o*-phthaldialdehyde–treated amino acids.

Advanced Approach to Amino Acid Sample Preparation and GC Analysis

We have developed a simple and fast procedure for body fluid sample preparation that obviates the need for plasma protein precipitation and any SPE-eluent evaporation. It comprises three rapid steps:

- preisolation of amino acids on a strong cation exchanger,
- recovery of the isolated analytes with an extraction–reaction medium, and
- derivatization with phase transfer using an organic phase with an incorporated alkyl chloroformate reagent.

Briefly, we pass 100-μL or smaller samples of biological material (for example, plasma, serum, or urine) and the same volume of internal standard solution through a 40-μL resin bed packed in a pipette tip (52). After a washing step, we expel the ion exchanger into 200 μL of reaction medium. Subsequently, we add a derivatization reagent dissolved in an organic solvent twice, and the phase-transfer-mediated derivatization proceeds by repeated vortex mixing for approximately 3–5 s each. Finally, we add 100 μL of acid solution and mix the upper organic phase briefly, after which the solution is ready for GC analysis. The whole sample preparation process can be accomplished in 5–7 min. For the targeted profiling of selected analytes such as aromatic and sulfur-containing amino acids, the procedure can be simplified further with even shorter preparation times.

A relatively short capillary column (10 m) and a rapid temperature rise enable fast analysis times with baseline separation of more than 19 protein amino acids (without arginine) (see Figure 1). The analysis of homocysteine and some other important amino acids can be accomplished on the same column in 5 min (see Figure 2). Analysts can monitor the physiological levels of total plasma homocysteine (<10 μmol/L) by using flame ionization detection; however, they can markedly enhance the selectivity and detection limits of sulfur-containing analytes by using specific detectors such as MS or flame photometric detectors (53). Moreover, when using flame ionization detection, workers must identify a suitable GC column for the complete separation of complex mixtures; however, this problem is less severe with GC–MS equipment because selective monitoring of ion traces can differentiate particular analytes.

Advanced Approach to Amino Acid Analysis by LC–MS

The alkyl chloroformate–mediated derivatization of amines and amino acids is unique because it creates compounds amenable for both GC–MS and LC–MS analysis (54). Our current studies focus on optimizing the approach to the fast analysis of amino acids in various biological matrices. Thus, the body fluid sample preparation follows the procedure described above, except for the last step, which involves addition of an acid solution. All protein amino acids, including those with untouched functional groups — such as arginine, with a free guanidine group — can be determined by omitting it. Because of the excellent sensitivity of the derivatives in atmospheric-pressure ionization MS, only 5–10 μL of the organic layer is mixed with 10–20 μL of the LC mobile phase and directly subjected to LC–electrospray ionization MS analysis on a C8 reversed-phase column (Figure 3).

The Novel Approach on Trial

While reviewing current methodologies for amino acid analysis, we found remarkably modest improvements in the preparation of biological samples in recent years despite rapid development in concentration technologies such as SPE and also in new derivatization approaches such as those that use alkyl chloroformates (55). A rapid reaction with high yields in seconds and robust reaction conditions — for example, in the presence of inorganic salts — enables users to integrate the chloroformate derivatization approach into a liquid–liquid extraction procedure and to examine the possibility of treating body fluids directly. Earlier attempts using a fluorinated chloroformate with the same purpose were only partially successful (56). Minimizing the contamination of the analytical system with matrix

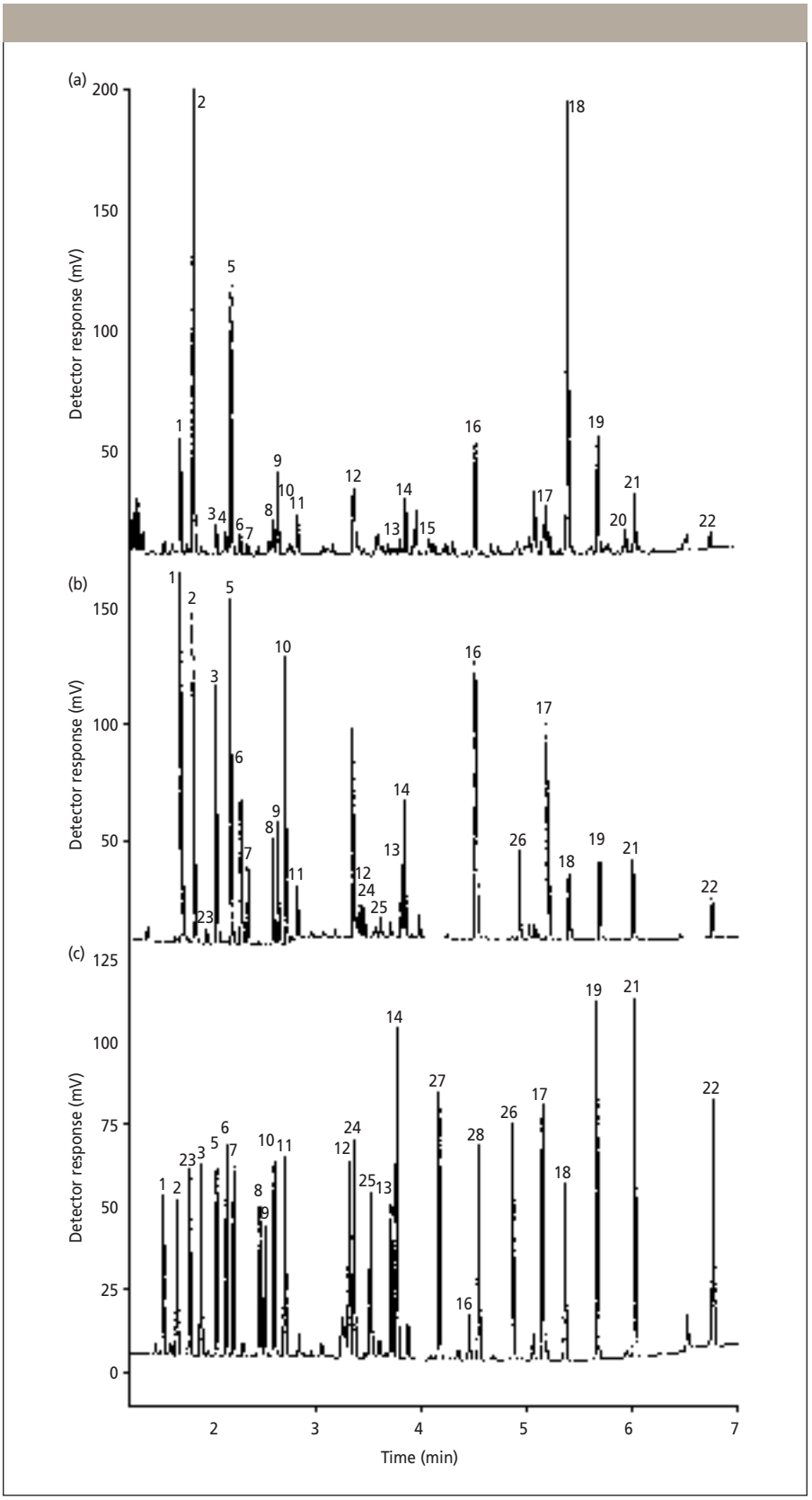

Figure 1: GC–flame ionization detection analysis of amino acids in 100 μL each of (a) urine and (b) serum and (c) amino acid standards (equimolar concentration). Column: Zebron Amino Acid GC; oven temperature program: 110–320 °C at 30 °C/min. Peaks: 1 = alanine, 2 = glycine, 3 = valine, 4 = β-aminoisobutyric acid, 5 = norvaline (internal standard), 6 = leucine, 7 = isoleucine, 8 = threonine, 9 = serine, 10 = proline, 11 = asparagine, 12 = aspartic acid, 13 = glutamic acid, 14 = phenylalanine, 15 = α-aminoadipic acid, 16 = glutamine, 17 = lysine, 18 = histidine, 19 = tyrosine, 20 = proline–hydroxyproline dipeptide, 21 = tryptophan, 22 = cystine, 23 = α-aminobutyric acid, 24 = methionine, 25 = hydroxyproline, 26 = ornithine, 27 = cysteine, 28 = homocysteine.

interferences such as lipids and fatty and hydroxy acids is highly desirable from a practical standpoint of avoiding undesired maintenance time and operating costs. To meet the demands for amino acid profile analysis, we have developed a simple combination of SPE and liquid–liquid extraction, without any extensive fluid pretreatment such as plasma protein precipitation.

We tested various sorbents, including AG 50W-X2, 50W-X4, and 50W-X8 cation exchangers (Bio-Rad Laboratories, Hercules, California) with 2–8% divinylbenzene (DVB) cross-linking; newly available materials based upon organic copolymers, such as Oasis HLB (Waters); some silica-based supports such as BondElut SCX (Varian, Inc., Palo Alto, California), and other sorbents for the SPE step. Our studies revealed that

- The amount of proteins captured with the amino acids and later released into the eluate varied from sorbent to sorbent and was mostly pH independent. Therefore, plasma samples without any pH adjustment proved to be perfectly suitable for analysis.
- In contrast with some previous reports, the uptake of amino compounds was unexpectedly fast and largely indepen-

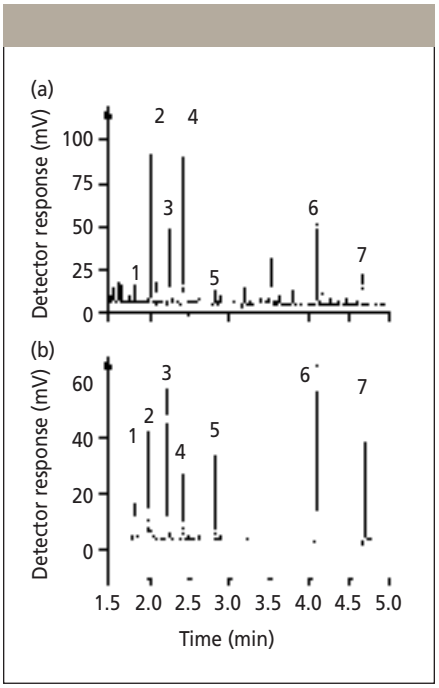

Figure 2: GC–flame ionization detection analysis of *S*-amino and aromatic acids (a) in human plasma and (b) corresponding standards. Column: Zebron Amino Acid GC; oven temperature range: 140–300 °C. Peaks: 1 = methionine, 2 = ethionine (internal standard at 200 μmol/L), 3 = phenylalanine, 4 = cysteine, 5 = homocysteine, 6 = tyrosine, 7 = tryptophan.

dent of the flow velocity through the sorbent bed. We observed no decrease in recovery even when the fluid penetrated the bed in the course of approximately 5 s.

- The affinity for aromatic amino acids increases with the degree of resin cross-linking, and their recoveries were correspondingly lower regardless of eluent composition (mostly ammonia based) (see Figure 4). To our knowledge, this aspect has been overlooked in previous reports in which the AG 50W-X8 type resin had been used almost exclusively.
- Commercial cartridges commonly used in earlier studies, which held sorbent amounts of 30 mg or more, were found to be too large, because with more sorbent, more eluent must evaporate. For competing matrix cations such as sodium in plasma and urea in urine, 5 mg of sorbent was sufficient to successfully deal with as much as 100 μL of the corresponding biological fluid.
- To dispense minute amounts of sorbent, we needed to develop a flexible technique for the preparation of miniature sorbent tips (52).
- A minute volume (≤ 200 μL) of alkaline medium, which was compatible with the subsequent chemical treatment, was equally effective for elution as the commonly used aqueous ammonia, so any evaporative step was redundant and unnecessary.

Additional studies were aimed at investigating whether the deproteinization step was necessary. If not, direct treatment would not only speed sample preparation but also eliminate inconsistencies related to the protein removal process. Protein precipitation in physiological fluids is considered a major problem in amino acid analysis, mainly because of the lack of standardized conditions (11). This problem was also confirmed in our own studies, when we noticed considerable variability in amino acid recoveries, depending upon the final concentration of trichloroacetic acid used in the protein precipitation step (often 10% instead of 3%) (see Figure 5).

Numerous experiments performed with commonly used deproteinization reagents under optimum conditions — 6% sulfosalicylic acid and 6% trichloroacetic acid with a 1:1 plasma ratio and acetonitrile with a 4:1 plasma ratio — showed that protein removal by precipitation was of no benefit compared with direct SPE of plasma samples. Recoveries varied no more than ±10% from the mean, except for the basic amino acids, especially in which case the recovery yields were substantially lower after acetonitrile-mediated precipitation (Figure 6). This observation is consistent with previous findings in which lysine was poorly recovered from plasma samples treated with acetonitrile (12). Because this solvent is used frequently as a deproteinizing agent before LC analysis, we wish to turn attention to this important point.

The subsequent phase transfer alkylation by liquid–liquid extraction proceeded smoothly and required only 3–5 s of repeated vortexing of the SPE eluent with an organic phase that contained the alkyl chloroformate reagent. Adding organic phase in two steps prevented cyclization of glutamic acid to pyroglutamic acid and enhanced the yields of some amino acids.

The total time required for body fluid amino acid profiling by the procedure described above is 15 min, including both sample preparation and GC analysis times. Along with the use of cost-effective GC–flame ionization detection equipment, the cost of the presented sample preparation is very low, which makes the procedure extremely economical. The treatment proved to be simple and robust; it was uninfluenced by the presence of salts and remaining proteins from the SPE step. We were able to directly subject protein hydrolyzates (6 M hydrochloric acid) to SPE in volumes as large as 10 μL after neutralization with bicarbonate. The only shortcoming of the procedure is its inability to detect arginine and citrulline because of an inefficient alkylation of the guanidine (ureic) group.

Comparative studies using a classic amino acid analyzer and based upon self-

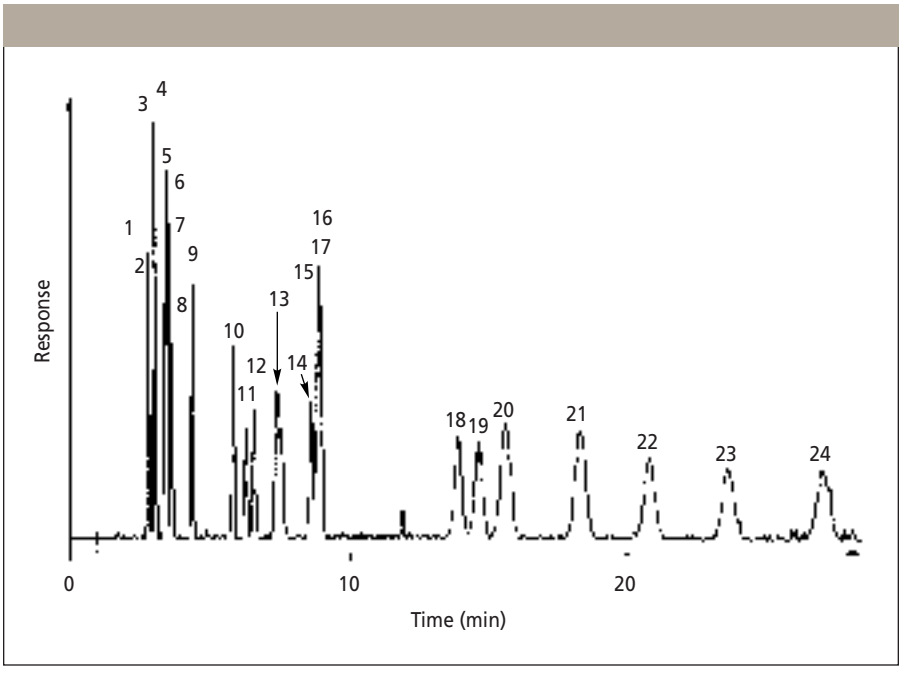

Figure 3: Reconstructed ion chromatogram of the LC–electrospray ionization MS analysis of an equimolar mixture of amino acid standards. Column: 150 mm × 1 mm C8; mobile phase: aqueous methanol buffered with 10 mM ammonium formate; flow rate: 50 μL/min; ionization: electrospray. Peaks: 1 = arginine, 2 = glutamine, 3 = citrulline, 4 = serine, 5 = hydroxyproline, 6 = glycine, 7 = asparagine, 8 = threonine, 9 = alanine, 10 = proline, 11 = aspartic acid, 12 = ornithine, 13 = histidine, 14 = glutamic acid, 15 = methionine, 16 = valine, 17 = lysine, 18 = cysteine, 19 = isoleucine, 20 = leucine, 21 = phenylalanine, 22 = cystine, 23 = tryptophan, 24 = tyrosine.

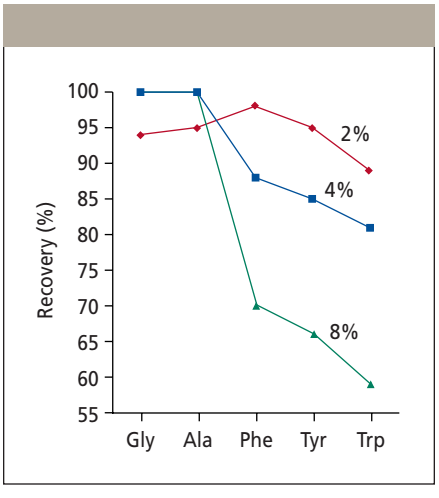

Figure 4: Recoveries of individual amino acids after SPE on a strong cation exchanger with different degrees of DVB cross-linking (2, 4, and 8%).

validating protocols meant to ensure reproducibility in recoveries and precision revealed a high level of method agreement and deviations less than ±10%. The sample preparation also enables LC–MS analysis of the same amino acid derivatives with the supplemental advantage of excellent atmospheric-pressure ionization MS sensitivity and ability to detect all protein amino acids, including arginine. To make the novel preparation accessible for routine use, we developed a kit in collaboration with Phenomenex Inc. (Torrance, California), with the commercial name of EZ:faast amino acid analysis kit.

Conclusion

A brief review of the latest trends in amino acid analysis reveals that sample preparation remains a crucial step in any methodology and that any real progress made in sample preparation can affect a particular approach more significantly than using complex and expensive instrumentation. Our studies show that users obtain top performance, even with an economical and multiple-use instrument such as a GC–flame ionization detection system, by using expedient sample preparation. Moreover, potential users may choose the same sample preparation procedure with other instruments available in their laboratories such as GC–MS or LC–MS systems. With the latter techniques, analysts can quantify subpicomole-level amino acids without making any changes in sample pretreatment. Researchers making any future advances in body fluid amino acid analyses should consider the advantages of the method presented in this article with regard to speed, simplicity, accuracy, and cost effectiveness.

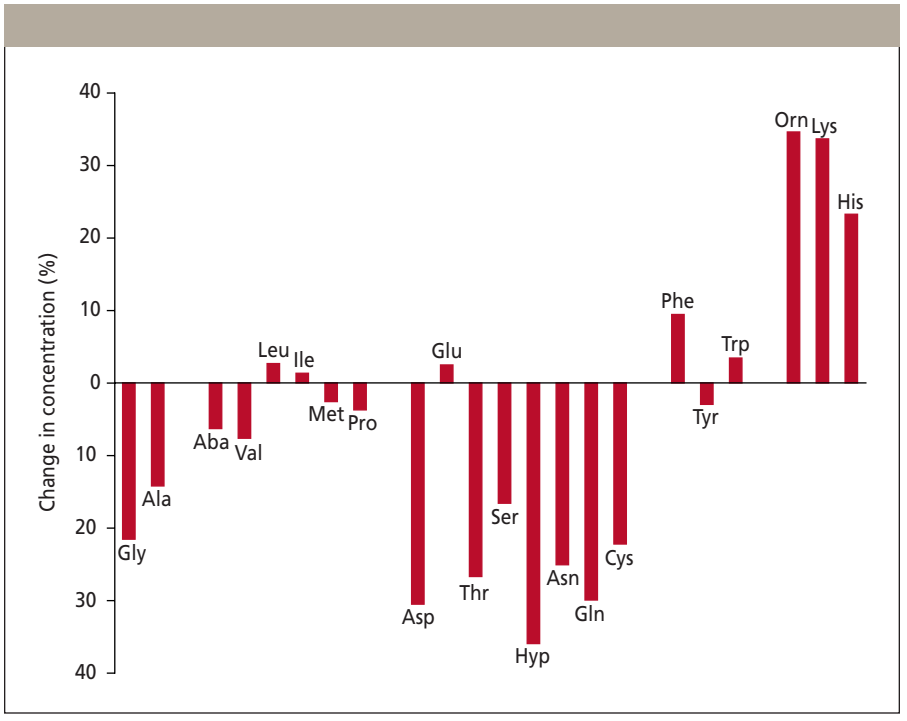

Figure 5: Changes in plasma amino acid yields after deproteinizing plasma with 10% and 3% ($y = 0$) aqueous trichloroacetic acid. Values on the y axis represent altered yields found with the use of 10% trichloroacetic acid compared with 3% trichloroacetic acid.

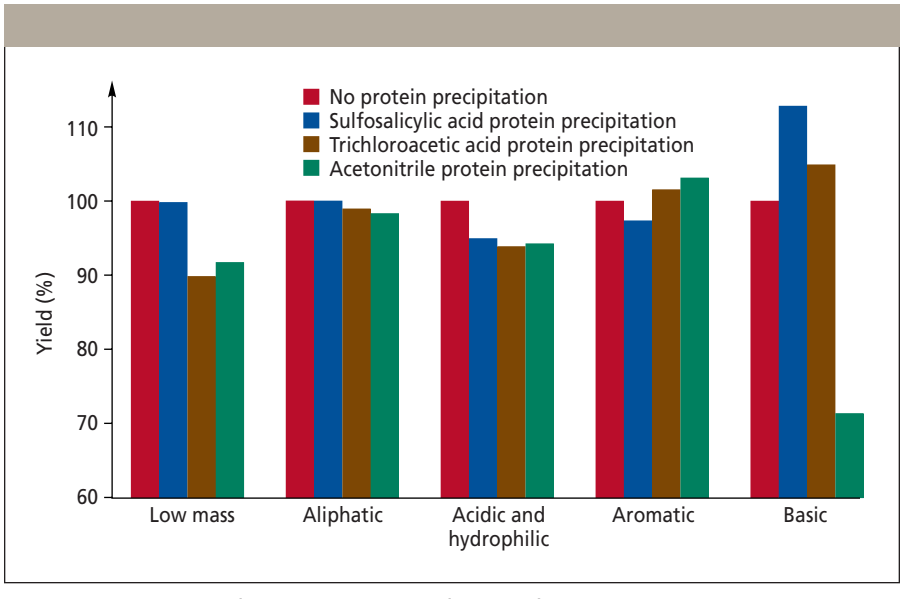

Figure 6: Recoveries of plasma amino acids after SPE of plasma samples.

Acknowledgments

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